Localization of von Willebrand Factor-binding Sites for Platelet Glycoprotein Ib and Botrocetin by Charged-to-Alanine Scanning Mutagenesis*

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Abstract

At sites of vascular injury, von Willebrand factor (VWF) mediates platelet adhesion through binding to platelet glycoprotein Ib (GPIb). Previous studies identified clusters of charged residues within VWF domain A1 that were involved in binding GPIb or botrocetin. The contribution of 28 specific residues within these clusters was analyzed by mutating single amino acids to alanine. Binding to a panel of six conformation-dependent monoclonal antibodies was decreased by mutations at Asp514, Asp520, Arg552, and Arg611 (numbered from the N-terminal Ser of the mature processed VWF), suggesting that these residues are necessary for domain A1 folding. Binding of 125I-botrocetin was decreased by mutations at Arg629, Arg632, Arg636, and Lys667. Ristocetin-induced and botrocetin-induced binding to GPIb both were decreased by mutations at Lys599, Arg629, and Arg632; among this group the K599A mutant was unique because 125I-botrocetin binding was normal, suggesting that Lys599 interacts directly with GPIb. Ristocetin and botrocetin actions on VWF were dissociated readily by mutagenesis. Ristocetin-induced binding to GPIb was reduced selectively by substitutions at positions Lys534, Arg271, Lys272, Glu497, Glu511, Arg416, Glu497, and Lys642, whereas botrocetin-induced binding to GPIb was decreased selectively by mutations at Arg611, Arg616, and Lys667. The binding of monoclonal antibody B24 induced Lys660 and Arg663, and this antibody inhibits 125I-botrocetin binding to VWF. The crystal structure of the A1 domain suggests that the botrocetin-binding site overlaps the monoclonal antibody B24 epitope on helix 5 and spans helices 4 and 5. The binding of botrocetin also activates the nearby VWF-binding site for GPIb that involves Lys599 on helix 3.

ADHESION OF PLATELETS TO VESSEL WALLS IS A FIRST STEP IN NORMAL HEMOSTASIS AND ALSO IN THROMBOTIC EVENTS SUCH AS THE OCCLUSION OF ATEROSCLEROTIC ARTERIES. THESE EVENTS ARE MEDITATED BY AT LEAST TWO LIGAND-RECEPTOR INTERACTIONS: VON WILLEBRAND FACTOR (VWF) BINDING TO PLATELET GLYCOPROTEIN Ib (GPIb) AND FIBRINOGEN BINDING TO PLATELET GPIIb-IIIa. VWF DOES NOT BIND SPONTANEOUSLY TO PLATELETS IN BLOOD, AND ITS ADHESIVE PROPERTIES ARE INDUCED IN VIVO UPON THE BINDING OF VWF TO SUBENDOTHELIAL CONNECTIVE TISSUE, PARTICULARLY UNDER CONDITIONS OF HIGH SHEAR STRESS THAT OCCUR IN THE MICROCECULATION.

VWF CONSISTS OF DISULFIDE-LINKED MULTIMERS THAT ARE ASSEMBLED FROM SUBUNITS OF ~250 KDA. THE VWF MULTIMERS RANGE IN SIZE FROM DIMERS OF ~500 KD TO >10,000 KD. VWF BINDS TO AN N-TERMINAL DOMAIN OF THE GPIbα SUBUNIT (2–4), AND THE GPIb-binding site on VWF CORRESPONDS APPROXIMATELY TO THE FIRST OF THREE REPEATED A DOMAINS IN THE VWF SUBUNIT. DOMAIN A1 EXTENDS FROM GLU497 TO GLY516 AND CONTAINS AN INTRACHAIN DISULFIDE LOOP THAT IS DEFINED BY THE DISULFIDE BOND CYS509–CYS505 (5, 6). BINDING OF VWF TO GPIb IN VITRO CAN BE INDUCED BY THE ANTIBIOTIC RISTOCETIN OR BY THE SNAKE VENOM PROTEIN BOTROCETIN. RISTOCETIN APPARENTLY CAN BIND BOTH TO PLATELETS AND TO VWF (7), WHEREAS BOTROCETIN BINDS TO VWF DOMAIN A1 BUT NOT TO GPIb (8).

By clustered charged-to-alanine scanning mutagenesis, we have found several clustered mutants of charged residues within VWF-A1 domain showing reduced or increased function (9). Several mutations in the two acidic segments Glu497–Arg511 and Arg687–Val698, which contain the Cys509–Cys505 disulfide, resulted in decreased binding to GPIb (9). In addition, mutations within discontinuous segments including Glu497–Arg616, Arg619–Arg632, and Lys642–Lys645 decreased binding to GPIb, suggesting that several residues within these segments may interact with GPIb. We now have prepared 28 new mutants in which a single charged residue is changed to alanine, and these proteins were used to characterize the binding sites for botrocetin and GPIb. The results suggest that botrocetin and GPIb bind to adjacent sites on the VWF-A1 domain, and the amino acid residues required for ristocetin-induced binding to GPIb are distinct from those required for botrocetin-induced binding.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were obtained from New England BioLabs (Beverly, MA). Taq DNA polymerase was from Perkin-Elmer. Highly purified two-chain botrocetin was provided by Dr. Yoshihiro Fujimura (Nara Medical University, Nara, Japan). Monoclonal antibody 6D1 against human platelet GPIb (10) was provided by Dr. Barry Collier (Mt. Sinai Medical Center, NY). Anti-VWF monoclonal antibody 33E12 was provided by Dr. Claudine Mazurier (Centre Regional de Transfusion Sanguine, Lille, France).

Monoclonal antibodies (mAbs) recognizing the VWF-A1 domain were kindly provided by the following researchers: NMC-4 (Dr. Midori Fujimura, Nara Medical University, Nara, Japan). Monoclonal antibody 6D1 against human platelet GPIb (10) was provided by Dr. Barry Collier (Mt. Sinai Medical Center, NY). Anti-VWF monoclonal antibody 33E12 was provided by Dr. Claudine Mazurier (Centre Regional de Transfusion Sanguine, Lille, France).

Monoclonal antibodies (mAbs) recognizing the VWF-A1 domain were kindly provided by the following researchers: NMC-4 (Dr. Midori Fujimura, Nara Medical University, Nara, Japan), AvWF3 (Dr. Philip Kroner, The Blood Center of Southeastern Wisconsin), CLB-RAG34 and CLB-RAG55 (Dr. J. A. van Mourik, Netherlands Red Cross Blood...
Expression and Characterization of Recombinant VWF—Human 293T cells (12) were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Cells were transfected by a calcium-phosphate method and recombinant VWF (rVWF) secreted in the medium was harvested and concentrated by ultrafiltration as described (9). VWF antigen was measured by an ELISA using polyclonal rabbit anti-human VWF antibody 082 and peroxidase-conjugated rabbit anti-human VWF antibody 226 (DAKO) (13). Multimer analysis was performed as described (14).

Binding of VWF to mAb Panel—The IgG fractions of mAbs NMC-4, AVWF3, 52K, CLB-RAG34, and CLB-RAG35 were isolated from ascites fluid by chromatography on recombinant protein A agarose (Amerham Pharmacia Biotech). Binding was assessed by ELISA using microtiter plates with U-shaped bottoms (Coster, Cambridge, MA). Plates were coated with 25 μl of each mAb (7.5 μg/ml) in 0.1 M sodium carbonate, pH 9.6, for 24 h at 4 °C. The wells were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 and then incubated with various concentrations of wild type or mutant rVWF diluted in PBS containing 3% bovine serum albumin (Sigma). The wells were washed again and incubated with antibody 226 diluted in PBS containing 3% bovine serum albumin color development with o-phenylenediamine, and the absorbance at 490 nm was determined. Binding of rVWF mutants was determined at a fixed concentration of rVWF (500 ng/ml) and normalized to the value obtained for wild type rVWF in paired assays. Negative control assays were performed by using concentrated conditioned medium from mock-transfected 293T cells.

125I-Botrocetin Binding Assays—Botrocetin binding to VWF was assayed according to methods of Fujimura et al. (15) as described previously (9). The assay was performed using anti-VWF mAb 3E12 that binds to the C-terminal region of the VWF subunit. It has no effect on VWF binding to platelets in the presence of either ristocetin or botrocetin (16). In brief, botrocetin was radiiodinated with 125I using a chloramine-T method (17). Microtiter plates were coated with 25 μl of 3E12 (7.5 μg/ml) for 16 h at 4 °C. The wells were washed, blocked, and incubated with 15 μl of each rVWF mutant (5 μg/ml) for 3 h at room temperature. The wells were washed again, and 5 μl of 125I-labeled botrocetin solution was added for 30 min at room temperature. Following rapid washing, air dried wells were excised and the bound radioactivity was measured by γ spectrophotometry. Nonspecific binding was obtained by assaying culture supernatant from mock-transfected cells, and specific binding was calculated by subtracting nonspecific from total binding. Values for mutant proteins at 3 μg/ml of 125I-botrocetin were normalized to paired values for wild type rVWF.

mAb B724 was assayed for ability to inhibit 125I-botrocetin binding to VWF. Microtiter plates coated with mAb 3E12 were incubated with wild type rVWF or mutant K599A. 125I-botrocetin (3 μg/ml), and the indicated amount of mAb B724 or control mouse IgG (DAKO) was added and then incubated for 30 min at room temperature. After washing, the bound radioactivity was measured as described above. The observed botrocetin binding was normalized to the value obtained in the absence of competing IgG.

Platelet Binding Assays—Assays were performed as described previously (9). To assay botrocetin-induced binding of VWF to GPIb, 570 ng/ml of rVWF was mixed with 2 × 10^6/ml of lyophilized human platelets (Biodata, Hatboro, PA) and incubated with various concentrations (0–10 μg/ml) of botrocetin. After 30 min at room temperature, the VWF antigen present in the centrifuged supernatant was measured by ELISA. Data for botrocetin concentrations bracketing the midpoint of the dose response curve (2, 3, and 6 μg/ml) were pooled for statistical analysis. To assay ristocetin-induced binding of VWF to GPIb, 500 ng/ml of rVWF was mixed with 2 × 10^6/ml of platelets, 4% bovine serum albumin, and various concentrations (1.5–5 mg/ml) of ristocetin (KabiPharmaceu- ticals, Stockholm, Sweden). After 30 min at room temperature, the VWF in the centrifuged supernatant was measured by ELISA. Reaction mixtures without platelets were tested simultaneously to verify the absence of nonspecific VWF flocculation and sedimentation in the presence of ristocetin. The unbound VWF was expressed as a percentage of the values obtained with no modulators, and the percentage of bound VWF was calculated by subtraction from 100%. The percentage of bound VWF was normalized to the paired value obtained for wild type rVWF assayed concurrently. Data at 1.5 mg/ml ristocetin were used for statistical analysis.

Statistical Analysis—Means and S.D. were calculated by standard methods. Confidence intervals (p < 0.05) for the means were estimated using the t distribution where the interval is given by the means ± S.D. × t(n−1)/2.

Crystallographic Structure Representations—Connelly surface plots of recombinant VWF A1 domain were prepared with the program INSIGHT II (Molecular Simulations, San Diego, CA). Schematic drawings of secondary structure elements of domain A1 were prepared with the program MOLSCRIPT (18).

RESULTS

Design and Expression of VWF A1 Domain Mutants—In a previous study by charged-to-alanine mutagenesis, we identified 6 single mutants and 13 clustered mutants with defects in binding to either botrocetin or platelet GPIb. From these data, the binding site for botrocetin or GPIb required amino acid residues within four segments of the VWF-A1 domain: 514–534, 549–552, 596–645, and 663–667 (Fig. 1) (9). Other reports suggested that binding to GPIb and heparin required residues within the segment 569–573 (19), and preliminary studies indicated that monoclonal antibody B724 (20) did not bind to construct (656–660)2A.

We further studied these six segments by constructing 28 additional single charged-to-alanine mutations (Fig. 1). Human kidney 293T cells were transfected with each mutant construct, and serum-free media were analyzed for the expression of rVWF. All 28 mutants were expressed at detectable levels and secreted efficiently. The multimer distribution of all mutant proteins was similar to that of wild type rVWF and plasma VWF; in every case, at least 12 multimer bands were detected (data not shown).

rVWF Binding to Monoclonal Antibodies—The folding of the A1 domain with each rVWF protein was evaluated with a panel of six conformation-dependent monoclonal antibodies: NMC-4...
FIG. 2. Binding of rVWF to monoclonal antibodies against the human VWF-A1 domain. Each monoclonal antibody (indicated at the top of each histogram) was coated onto plastic microtiter wells. Binding was determined at a fixed concentration of rVWF (500 ng/ml) and normalized to the value obtained for wild type rVWF as described under “Experimental Procedures.” Except for 52K2, all the antibodies bind to unreduced but not to reduced VWF A1 domain. 52K2 reacts with both reduced and unreduced VWF. The mutant rVWF proteins are indicated at the left. Each column represents the mean and S.D. obtained for at least duplicate assays. The results of NMC4 binding for 33 mutants were reported previously (9), and for comparison these results are indicated by open columns, representing the mean ± S.D. of values obtained in at least two independent sets of duplicate assays. Asterisks indicate values that are significantly different from 100% (p < 0.05).

FIG. 3. Binding of VWF to mAb B724. mAb B724 was immobilized on microtiter plates, and VWF binding was determined as described under “Experimental Procedures” for wild type rVWF (closed circles), mutant K660A (open circles), and mutant R663A (closed squares). Non-specific binding (open squares) was determined with concentrated conditioned medium from mock-transfected 293T cells and was undetectable. Each data point represents the mean ± S.D. of values obtained in at least two independent sets of duplicate assays. The symbols are larger than the S.D. range in most cases.

(21, 22), AvWF3 (23), CLB-RAG34, CLB-RAG35 (24), B724 (20), and 211A6 (25). Recognition of domain A1 by these antibodies is impaired by reduction or denaturation. Antibody 52K2 reacts with both reduced and nonreduced forms of VWF (21) and was used as a control. For each antibody, the absorbance value obtained for each mutant rVWF was expressed as a percentage of the value obtained for wild type rVWF (Fig. 2). All rVWF proteins displayed normal binding to mAb 52K2. Concentrated conditioned medium from mock transfected cells gave no signal with any of these antibodies.

Mutants D514A, D520A, R552A, and R611A showed markedly decreased binding to all six conformation-dependent mAbs (Fig. 2), suggesting that these substitutions cause significant misfolding of the A1 domain. Therefore, although further ligand binding data were obtained for these proteins, the results were excluded from additional interpretation. In fact, the binding of this class of mutants to botrocetin and GPIb was reduced >70% in all assays (data not shown). Mutant K534A showed decreased binding to four mAbs (AvWF3, CLB-RAG34, CLB-RAG35, and B724), although it retained nearly normal binding to two others (NMC-4 and 211A6). Thus, Lys534 is at least required for the presentation of epitopes for several antibodies and may be important to maintain the normal conformation of the A1 domain.

Other substitutions caused relatively selective defects in mAb binding. For example, at a concentration of 0.5 μg/ml mutant rVWF, binding to mAb B724 was decreased markedly by the mutations K660A and R663A (Fig. 2). These mutants showed normal binding to all other antibodies. Dose response binding curves illustrate the substantial decrease in affinity caused by the substitution K660A or R663A (Fig. 3) and suggest that the epitope of mAb B724 includes the side chains of Lys660 and Arg663. Similarly, binding of mAb NMC4 was reduced selectively by the substitution R632A, suggesting that its epitope contains the side chain of Arg632.

Binding of Botrocetin to rVWF—Previous charged-to-alanine mutagenesis studies suggested that several amino acid residues of VWF domain A1 participate directly in binding to 125I-botrocetin, including Arg632, Arg636, and residues in the segments Lys642–Lys645 and Arg663–Lys667 (9). In addition, clustered mutations in other segments decreased binding to
Botrocetin, but their significance was uncertain because these mutations also impaired binding to mAb NMC4 so that protein misfolding could not be excluded (9).

To address this uncertainty, additional rVWF mutants were constructed and assayed for binding to 3 μg/ml 125I-botrocetin (Fig. 4). At this concentration of botrocetin, binding to VWF is approximately half of the maximum (9, 15). Four previously constructed proteins that had shown decreased binding to GPIb or botrocetin were included as well: K534A, E626A, R632A, and R636A.

Four mutants exhibited decreased binding <60% of wild type rVWF: R629A, R632A, R636A, and K667A. As discussed below, the mutations R629A and R632A also disrupt binding to GPIb induced by ristocetin, whereas the mutations R636A and K667A do not. Therefore, the side chains of Arg636 and Lys667 are specifically required for binding to botrocetin. As reported previously for plasma VWF (20), mAb B724 inhibited the binding of botrocetin to immobilized wild type rVWF (data not shown).

Although botrocetin binding was markedly reduced by the clustered mutation of four lysine residues in construct (642–645)A (9), the individual mutation of each lysine had a modest effect on binding to botrocetin (Fig. 4), suggesting that no one of these residues makes a substantial contribution to this interaction. Similarly, the clustered mutant (613–616)A exhibited markedly impaired binding to botrocetin (9), but the corresponding single mutants E613A and R616A bound with slightly decreased affinity to botrocetin (Fig. 4).

Binding of rVWF to GPIb—Binding to GPIb was assessed by quantitating the rVWF that bound to formalin-fixed platelets in the presence of ristocetin (1.5 mg/ml) or botrocetin (2–6 μg/ml). In either case, binding to platelets was blocked completely by a mAb 6D1 to platelet GPIb (10) as reported previously (26). Values for each mutant protein were normalized to the values obtained for wild type rVWF as described under “Experimental Procedures.” Data for five mutants (D514A, K534A, E626A, R632A, and R636A) were reported previously (9). Each bar represents the mean ± S.D. of values obtained for up to four independent assays. Asterisks indicate values that are significantly different from 100% (p < 0.05).

The mutants R636A and K667A showed decreased botrocetin-induced GPIb binding (Fig. 5) that is consistent with their decreased binding to 125I-botrocetin (Fig. 4). However, these proteins retained normal ristocetin-induced GPIb binding (Fig. 5), indicating that the side chains of Arg636 and Lys667 interact with botrocetin but not with GPIb.

Alanine substitutions at eight residues (Lys534, Arg571, Lys572, Glu596, Glu613, Arg616, Glu626, and Lys642) decreased ristocetin-induced GPIb binding without affecting botrocetin-induced GPIb binding (Fig. 5) or 125I-botrocetin binding (Fig. 4). Therefore, these sites appear to be required for ristocetin-dependent VWF modulation but not for direct interaction with GPIb.

Mutation at a residue that directly interacts with GPIb would be predicted to reduce both ristocetin-induced and botrocetin-induced GPIb binding, and three mutants had this phenotype: K599A, R629A, and R632A. Among them, only K599A retained normal binding to 125I-botrocetin. Because GPIb and botrocetin can bind simultaneously to VWF, the selective loss of GPIb binding suggests that Lys599 interacts directly with GPIb and not with botrocetin. The mutants R629A and R632A decreased binding to both GPIb and 125I-botrocetin, suggesting that these amino acid side chains could be involved in the binding of both ligands.

**DISCUSSION**

Crystallographic structures were determined recently for two forms of the VWF A1 domain (27, 28). The A1 domain contains a central β-sheet of five parallel strands and one antiparallel strand sandwiched between three α-helices on
each side (Fig. 6). A disulfide bond at one end of the β-sheet links the N-terminal and C-terminal ends, and the sequence flanking the disulfide bond at the N terminus of the domain lies across its lower surface. These structures provide a framework for interpreting the effects of charged-to-alanine mutations on ligand binding.

**Binding Sites for GPIb, Botrocetin, and Monoclonal Antibodies**—K599A was the only VWF A1 mutant that retained normal binding to botrocetin and lost both ristocetin-induced and botrocetin-induced binding to GPIb. This specific effect suggests that the GPIb-binding site involves Lys599, which is located in the middle of helix 3 (Fig. 6). The mutations R629A and R632A in the adjacent helix 4 impaired binding to GPIb but also decreased botrocetin binding, suggesting that Arg629 or Arg632 may interact with both GPIb and botrocetin. However, the interpretation of this dual defect is complicated by the strong positive cooperativity between botrocetin and GPIb binding. Because of this linkage, a single mutation in domain A1 might reduce the affinity for both ligands by interfering with allosteric changes rather than by disrupting a common binding site. The mutations R636A and K667A have a simpler phenotype, impairing botrocetin binding (Fig. 4) but not ristocetin-induced binding to GPIb (Fig. 5). The selective effect of these mutations indicates that the botrocetin-binding site is, in fact, adjacent to the GPIb-binding site and spans both helix 4 and helix 5 (Fig. 6).

**Fig. 6.** Ligand-binding sites on the VWF A1 domain. On the left is a surface representation of the VWF A1 domain based on the coordinates of Emsley et al. (28). The locations are indicated of amino acid residues involved in the binding of platelet GPIbα (red), mAb NMC-4 (green), and mAb B724 (blue). Residue Arg629 is colored in both green and orange to indicate that it interacts with both NMC-4 and botrocetin. On the right is a schematic drawing of the A1 domain in the same orientation. The coloring of amino acid side chains is the same as in the left panel. Only a partial side chain is shown for Lys660 because atoms more distal than Cα were not present in the structure (28). Helices are colored for clarity: α3 in red, α4 in orange, and α5 in blue. Helices are numbered according to Celikel et al. (27). The Cys509–Cys695 disulfide bond is shown at the bottom right, with S atoms in yellow.

**Fig. 7.** Location of amino acid residues required for ristocetin-induced binding of VWF to platelet GPIb. The stereo schematic drawing of the VWF A1 domain shows in yellow the location of amino acid side chains that, when mutated to alanine, result in the selective loss of ristocetin-induced binding to platelets. Side chains in blue indicate positions of mutations in patients with VWD type 2M that have been characterized by expression of recombinant mutant VWF. For reference, the side chain of Lys599 (red) is shown to indicate the approximate position of the binding site for GPIb.

**Fig. 8.** VWD type 2B gain-of-function mutations. A, the left panel is a surface representation of the VWF A1 domain showing the locations of mutations that cause VWD type 2B (yellow) and the location of K599 in the GPIb-binding site (red). In the right panel, the A1 domain has been rotated 90° about the vertical axis. B, this stereo schematic drawing is in the same orientation as the left panel of A. The side chains are shown and numbered for amino acid residues that are mutated in patients with VWD type 2B. Blue, nitrogen; red, oxygen; yellow, sulfur; black, carbon.
proximity of the NMC-4 epitope to the proposed GPIb site involving Lys<sup>642</sup> also is consistent with the ability of NMC-4 to inhibit the binding of VWF to platelets (22, 29).

In contrast to NMC-4, antibody B724 does not prevent the binding of VWF to platelets in the presence of ristocetin (20), suggesting that its epitope is distant from the GPIb site but close to the botrocetin site. This conclusion is supported by the results of mutagenesis (Fig. 2), which indicate that the B724 epitope contains residues Lys<sup>660</sup> and Arg<sup>663</sup> on helix 5 (Fig. 6). The adjacent residue Lys<sup>667</sup> contributes to the botrocetin-binding site and probably accounts for the ability of B724 to inhibit botrocetin binding to VWF (20).

These results suggest a model for the location of several binding sites on the VWF A1 domain (Fig. 6). Platelet GPIb<sub>a</sub> interacts with a site that includes Lys<sup>599</sup> within helix 3. Botrocetin binding requires residues in both helices 4 and 5 that are within or adjacent to the epitopes for NMC-4 and B724, respectively. NMC-4 binds to helix 4 at a site that is sufficiently close to the botrocetin site to prevent the binding of VWF to platelets. Antibody B724 binds to helix 5 at a site too remote to interfere with GPIb binding. All of these binding sites involve helices that are on the same side of the central β-sheet within the VWF A1 domain.

Selective Loss of Ristocetin-induced Binding to GPIb—Mutations that abolish the modulation of VWF activity by ristocetin frequently preserve modulation by botrocetin, and this phenotype indicates that ristocetin and botrocetin promote VWF binding to GPIb by fundamentally different mechanisms. Eight VWF mutations (K554A, R571A, K572A, E596A, E613A, R616A, E626A, and K642A) caused a selective decrease in ristocetin-induced binding to platelets. A few patients with severe bleeding and von Willebrand disease type 2M have plasma VWF with a similar phenotype. In three such cases, mutations within the A1 domain have been characterized by expression studies: G561S, F606I, and I662F (26, 30). In other cases, mutations appear to mark the location of a regulatory site that normally inhibits the binding of domain A1 to GPIb<sub>a</sub>. Mutations affecting the regulatory site can relieve this inhibition and cause the constitutive binding that characterizes VWD type 2B (9, 36, 37). A challenge for the future is to determine how mutations on one side of domain A1 can activate a GB1-binding site on the opposite side and whether this apparently allosteric regulation of VWF-platelet interactions is important for normal hemostasis.

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